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Evaluation of the potential role of water in spread of conidia of the *Neotyphodium* endophyte of *Poa ampla*

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ABSTRACT

Neotyphodium endophytes are asexual, filamentous fungi, mutualistically associated with diverse cool season grasses. Infected seeds and vegetative organs of infected host plants are the only known modes of propagation of the asexual endophytes. In the last decade certain *Epichloë* and *Neotyphodium*-infected grass species have been shown to have epiphyllous structures of the endophytes, hyphae, conidiophores, and conidia, growing on leaf blades. The production of epiphyllous conidia suggests the possibility that some of these endophytes may have the ability for plant-to-plant spread. The objective of this study was to determine the possible mechanisms involved in liberation and dispersal of asexual spores of *Neotyphodium* growing *in vitro* and epiphyllously on leaves of *Poa ampla*. Our results indicate that water dispersal is the most likely means of dissemination of conidia of the *Neotyphodium* sp. Wind generated by dry compressed air does not dislodge the conidia from slide cultures or from *P. ampla* leaves.

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Introduction

Endophytes of the genera *Epichloë* and *Neotyphodium* (*Clavicipitaceae*, *Hypocreales*) (Glenn *et al.* 1996; Schardl & Phillips 1997) are mutualistic fungi that colonize many cool season grasses of the subfamily *Pooideae*. Numerous studies of endophyte-grass symbiosis have documented that endophytes can influence host plant response to environmental stresses, increase host competitive ability, and provide other benefits to host plants, including nutrient acquisition, and resistance to herbivores and parasites by production of bioactive metabolites (Arachevaleta *et al.* 1989; Bacon *et al.* 1977; Clay 1990, 1998; Clay & Holah 1999; Clay & Schardl 2002; Clement *et al.* 2005; Fletcher & Harvey 1981; Ford & Kirkpatrik 1989; Gwinn & Gavin 1992; Kimmons *et al.* 1990; Latch 1993; Malinowski & Belesky 2000; Malinowski *et al.* 1997; Schardl *et al.* 2004).

Neotyphodium endophytes are asexual, filamentous fungi that are evolutionarily derived from sexual relatives, *Epichloë* species (Schardl *et al.* 1991). In the antagonistic association of plant with pathogenic *Epichloë*, the pathogens form a fungal fruiting structure (stroma) around the flag leaf sheath of the emerging inflorescence, which completely suppresses development of the grass inflorescence; this is referred to as choke disease. The sexual *Epichloë* pathogens can be transmitted horizontally through development of ascospores (Schardl *et al.* 1994; White 1988). Because *Epichloë* species are obligately out-crossing ascomycetes, development of the sexual spores is dependent upon transfer of spermatia of one mating type to an unfertilized stroma of the opposite mating type occurring on different individual host plants (White & Bultman 1987). Transfer of spermatia of *Epichloë typhina* is accomplished by flies of the genus *Botanophila* (*Anthomyiidae*, *Diptera*),

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which visit stromata for feeding and oviposition (Bultman & White 1988; Kohlmeyer & Kohlmeyer 1974; White & Bultman 1987). Immediately after cross-fertilization of the fungus, perithicia begin to develop in the stroma (Bultman *et al.* 1998). During flowering of the host plant, the ascospores produced within the perithecia of infected individuals in the population are forcibly ejected (Ingold 1948). The ascospores, possibly dispersed by air currents, land on another healthy grass plant inflorescence and may initiate infection (Chung & Schardl 1997a). In contrast, the mutualistic *Neotyphodium* endophytes of diverse cool season grasses do not produce obvious external structures. For most of their life cycle, they inhabit asymptomatically and systemically the apoplasts of the aboveground organs of infected host plant, including the embryos of viable seeds (Sampson 1933; Schardl 2001; Schardl & Phillips 1997; White 1988), and can be disseminated vertically. Infected seeds and vegetative organs of infected host plants are the only known modes of propagation of the asexual endophytes (Clay 1988; Schardl *et al.* 1994). In addition, several *Epichloë* spp., such as *Epichloë festucae*, are represented by species that have both horizontal and vertical transmission modes. In these cases, some tillers produce stromata on the same plant whereas other tillers produce infected seeds (White 1988).

Phyllosphere microbial communities are diverse; normally the phyllosphere is colonized by variety of different organisms, including bacteria, yeasts, and fungi (Andrews & Harris 2000). In the last decade certain *Epichloë* and *Neotyphodium*-infected grass species including *Agrostis hyemalis*, *Bromus setifolius*, *Hordeum brevisubulatum* subsp. *violaceum*, *Lolium pretense*, *Poa ampla*, *P. rigidifolia*, and several additional species have been shown to have epiphyllous structures of the endophytes, hyphae, conidiophores, and conidia, growing on leaf blades (e.g., Christensen *et al.* 1997; Craven *et al.* 2001; Dugan *et al.* 2002; Moon *et al.* 2002; Moy *et al.* 2000; White *et al.* 1996). The production of epiphyllic conidia suggests the possibility that some of these endophytes may have the ability for plant-to-plant spread using surface-produced conidia (White *et al.* 1996).

P. ampla (big bluegrass) colonized by *Neotyphodium* was selected for this study. *P. ampla* is a hardy, cool-season grass that is a native of western North America. It has been documented that, while colonizing *P. ampla*, *Neotyphodium* is also able to grow and develop mycelial structures with conidia on the surfaces of host plant leaves (Moy *et al.* 2000). The objective of this research was to determine the possible mechanisms involved in liberation and dispersal of asexual spores of *Neotyphodium* growing *in vitro* and epiphyllously on *P. ampla*.

Materials and methods

Plant and fungal material

Poa ampla samples infected by a *Neotyphodium* sp. were collected from sites along the Alaska Highway in Yukon, Canada. The plants were maintained in the Rutgers University Research Greenhouse Facility, in New Brunswick, New Jersey. Voucher material of the fungus used is preserved as a living culture in 20 % glycerol at −80 °C in the Rutgers University Plant Pathology Herbarium (RUTPP) at the School of Environmental and Biological Sciences, Rutgers University.

Segments of leaves of *P. ampla* in different developmental stages were used to confirm the presence of *Neotyphodium* on their surfaces. The leaves were cut from health plants maintained under greenhouse conditions and immediately examined. The examined material was stained and maintained according to procedures described in detail by White *et al.* (1996). In short, excised leaves were soaked in a solution of 0.1 % aniline blue in 85 % lactic acid for 5 min, removed from the stain, rinsed three times in a bath of sterile distilled water, and allowed to air dry. Three layers of colourless fingernail polish were applied to the surface of leaves tightly rolled around cylindrical scintillation vials. After complete drying, the fingernail polish layer was peeled from the surface of leaves and placed on slides. The slides were covered with mounting medium (Fisher Permount Mounting Medium, Fisher Scientific, Fair Lawn, NJ) and coverslips were then placed on the slides. The peels were examined for occurrence of fungal structures using a Nikon Labophot-2 compound light microscope (×150–600). Photographs of the peels and structures of the fungus were obtained with a Zeiss Axioskop light microscope with phase contrast and an Olympus CAMEDIA C-7000 ZOOM digital camera. For SEM the leaf fragments of *P. ampla* were mounted on stubs, sputter coated with gold–palladium and placed in the low-vacuum, variable-pressure chamber of the JEOL JSM 35C scanning electron microscope and photographed at 15 kV. Photographs and SEM images were processed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

Potato dextrose agar (PDA; Difco, Potato Dextrose Agar, Becton, Dickinson & Company, Sparks, MD) amended with 20 mg l^{-1} penicillin and 40 mg l^{-1} streptomycin sulphate (PDA + 2) was prepared and poured in Petri dishes (100 × 15 mm). A leaf wash method was used to isolate *Neotyphodium* sp. from the leaves of *P. ampla*. Point five millilitres of the leaf wash was pipetted onto each plate (PDA + 2). All plates were incubated in the dark at 22 °C. After 2–5 d typically slow-growing colonies of *Neotyphodium* sp. were transferred onto new PDA plates without antibiotics and incubated for 14 d. After 14 d of sub-culturing the culture was used to make a spore suspension with sterile distilled water. A 2 % agar block of PDA (25 × 30 × 3–4 mm) was placed on a glass slide. A suspension (0.03 ml) of conidia was then uniformly spread on the agar block. This slide culture was placed inside the sterile Petri dish and incubated in the dark for 10 d at 22 °C.

Preliminary air velocity experiments using conidia on slide cultures

In a preliminary experiment, a slide culture of *Neotyphodium* was placed on the stage of the compound light microscope and selected conidiophores bearing conidia were examined microscopically at ×60 magnification to determine whether conidia would be released by air. Compressed air from an airline in the laboratory was attached to a rubber hose. A 23-cm long Pasteur pipette (13-678-20D, Fisher Scientific, Pittsburgh, PA) in turn was attached to the rubber hose. The Pasteur pipette was positioned 3 cm from the slide culture. Compressed air was blown onto the *Neotyphodium* culture for 1 min. Four different air velocities were selected, 1.6, 3.5, 7.5, and

15.0 m s^{-1}, and measured at the level of slide culture with a pocket wind meter (Kestrel 1000, Nielsen Kellerman, Chester, PA). Mean air speed was calculated from three measurements recorded at 10 s intervals. Next, an aerosol sprayer (Spray Bottle FR-66-5565, Carolina Biological Supply Company, Burlington, NC) was filled with sterile distilled water. First, one squirt of water from the sprayer was applied onto the surface of a new slide culture of *Neotyphodium*. Immediately after the spray of water, the compressed air was released and blown onto the *Neotyphodium* culture for 1 min. Blown air or water droplets from the culture were collected onto Petri dishes (100 × 15 mm) containing PDA + 2. These Petri dishes will be referred to as trap plates. The trap plates were positioned 5 cm downwind of the culture. The dishes were then incubated at 22 °C in the dark and examined 2–5 d later to determine number of colonies. Three randomly selected microscopic fields on each trap plate were examined 24 h after inoculation under compound light microscope at ×60–300 to determine whether incipient colonies arose from conidia or from mycelial fragments. This experiment was done without replicates and data represent one experiment for each velocity and experimental design.

Experiments using conidia on slide cultures

A hand-held sprayer (Model GS, R&D Sprayers, Opelousas, LA) equipped with a nozzle (XR TeeJet 8003VS, Spraying System, Wheaton, IL) was attached to a gas cylinder (Dry Air, Airgas East, Cheshire, CT) with compressed air. The sprayer was clamped to a support stand. The operating pressure of air was 207 kPa with a velocity of 4.0 m s^{-1}. The distance between the nozzle and the slide culture of *Neotyphodium* was 30 cm. A slide culture was clamped in the support stand and positioned 10 cm above an opened trap plate containing a PDA + 2 medium. In the first part of this experiment, a stream of compressed air was directed towards the culture of *Neotyphodium* on the slide for 5 s. In the second part, a 2 l plastic container filled with sterile distilled water was attached to the sprayer to generate atomized water spray. A stream of atomized water spray was directed towards the *Neotyphodium* culture on the slide for 5 s. The coalescing water droplets on the slide culture were collected on one trap plate.

Experiments on epiphyllous conidia

Poa ampla leaf blades (ten blades, each *ca* 8 cm long) were used instead of the slide culture of *Neotyphodium*. The leaves were cut near the base of the blade and then bundled together with a piece of tape and immediately used in experiments. The same equipment (gas cylinder with compressed air, sprayer, nozzle, and 2 l plastic container) was used with the same pressure and air velocity as in the previous set of experiments. The distance between the nozzle and the cut portions of bundled leaves was fixed at 30 cm. The leaves were clamped to the support stand and positioned 10 cm above the opened plate containing the agar medium. In the first part of this experiment compressed air was directed towards some bundled leaves, while in the second part another bundle of leaves was treated with the atomized water spray defined above.

Cultural survey of epiphyllous fungi

The trap plates of the latter two experiments were incubated in the dark at 22 °C. On each trap plate, three randomly selected microscopic fields were examined after 24 h of incubation under the microscope at ×60–300 to determine whether incipient colonies arose from conidia or from hyphal fragments. After 2–5 d formation of *Neotyphodium* sp. and other fungal colonies were recorded. Slow-growing colonies of *Neotyphodium* sp., as well as colonies of other fungal species recovered from leaf surface of *P. ampla*, were transferred onto new PDA plates and incubated for the next 14 d. New slide cultures of *Neotyphodium* and fresh leaves of *P. ampla* were used for all three replicates. Data concerning these two experiments were presented as mean values of total number of colonies with standard deviation of three experiments.

Results

Presence of epiphyllous stage

The fungal structures produced on the PDA medium (Fig 1A–B) by *Neotyphodium* spp. isolated from leaf surfaces of *Poa ampla* were consistent with the structures produced by the fungus growing on the leaf surface of the host plant (Fig 1C–E). On both the agar medium and on leaf surfaces, tapering solitary conidiophores arose from hyphae. At the apex of conidiophores, the fusiform to lunate conidia were formed, and the conidia often formed the characteristic 'T-shape' at the conidiophore apex (Fig 1E).

Function of air and water in conidial dissemination

In the preliminary experiment (Table 1), slide cultures of *Neotyphodium* sp. were observed many times at ×60 magnification under the compound microscope when compressed air was blown for 1 min towards the conidiophores and conidia at the four different air velocities, 1.6, 3.5, 7.5, and 15.0 m s^{-1}. At all wind velocities every conidium remained steadfastly attached to its conidiophore during the 1 min when air was directed at the slide culture. At the higher air velocities (7.5 and 15.0 m s^{-1}), conidiophores at times would bend 45–60° from the vertical, and in some cases, the agar would pull away from the underlying slide. Yet even at the higher air velocities, no conidium became detached from its conidiophore. After the water spray was applied, no conidia were observed on conidiophores. In fact, the conidiophores appeared to be flattened and entwined with the hyphae on the slide culture when examined with the compound microscope. The trap plates confirmed the above observations. No *Neotyphodium* colonies appeared on the trap plates when only air was blown onto the slide cultures for 1 min. In contrast, numerous *Neotyphodium* colonies appeared on the trap plates when water from the sprayer was applied onto the surface of the slide cultures of *Neotyphodium* before the compressed air hit the slide cultures (Table 1). At all air velocities, numerous *Neotyphodium* colonies were observed after 2–5 d of incubation. Only conidia gave rise to colonies of *Neotyphodium*; no colonies were found to develop from hyphal fragments.

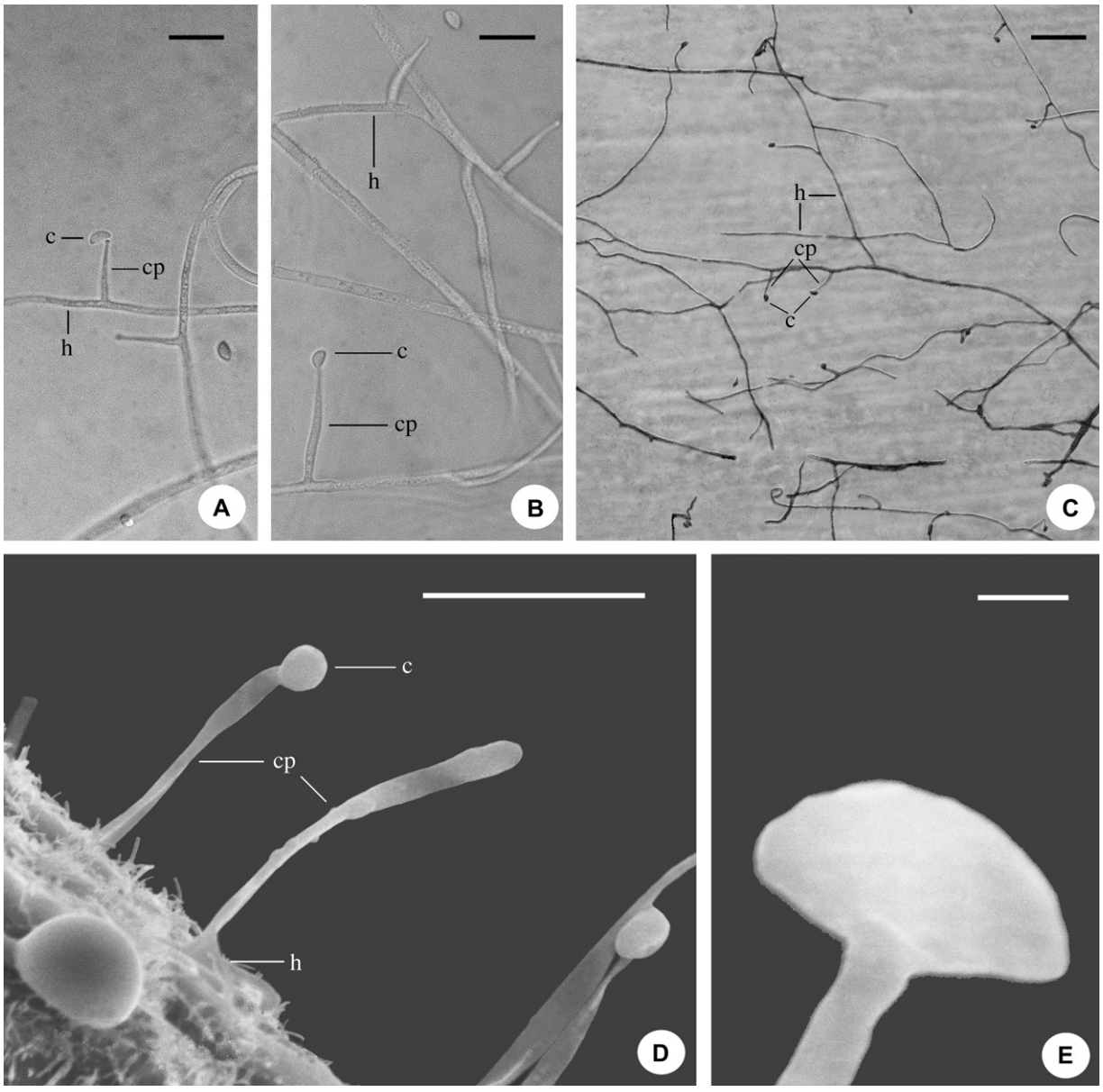

Fig 1 – *Neotyphodium* sp. on PDA medium (A–B) and on the leaf of *Poa ampla* (C–E). (A) Hyphae (h), conidiophore (cp) and mature conidium (c) of 14-d-old culture of *Neotyphodium*. Bar = 10 μm. (B) Hyphae (h), conidiophore (cp) and immature conidium (c) of 14-d-old culture of *Neotyphodium*. Bar = 10 μm. (C) Section of endophyte-infected leaf of *P. ampla* colonized by an epiphyllic stage showing a hyphal network (h), conidiophores (cp), and conidia (c). Bar = 20 μm. (D) Scanning electron micrograph of the fungus growing on the leaf surface of the host plant showing hyphae (h), conidiophores (cp), and conidium (c). Bar = 10 μm. (E) Scanning electron micrograph illustrating conidium of *Neotyphodium* on the apex of the conidiophore. Bar = 1 μm.

Similar results were obtained when compressed air from the gas cylinder was used (Table 2). No *Neotyphodium* colonies formed on the trap plates when only compressed air from the cylinder was blown onto the slide culture. However, an average of 61.3 colonies of *Neotyphodium* per trap plate were recorded when a water mist transported with compressed air fell on the slide cultures (Table 2).

When leaves of *Poa ampla* were blown with compressed dry air, no conidia of *Neotyphodium* sp. were trapped on plates. However, a few *Cladosporium* and yeast colonies were observed after incubation. When the atomized water spray was applied an average of 17.8 colonies of *Neotyphodium* were observed on trap plates after incubation (Table 3). Moreover, numerous colonies of other fungal genera were counted after the atomized water spray was applied. *Cladosporium* predominated with approximately 78 % of all colonies, while a lower percentage of other fungal genera, such as *Acremonium*, *Penicillium*, *Trichoderma*, yeasts, and sterile mycelium, were also found (Table 4).

Table 1 – Release of *Neotyphodium* conidia from slide cultures in preliminary experiment

Air velocity ($m\,s^{-1}$)	No. of colonies[a]	
	Air only	Air–water spray
1.6	0	658
3.5	0	672
7.5	0	705
15.0	0	778

a Data represent value of one experiment for each combination.

Discussion

Our results demonstrate that atomized water sprays release conidia of the *Neotyphodium* sp. grown on slide cultures. Although the number of *Neotyphodium* colonies in the second experiment (Table 2) was considerably lower than the number of colonies in the preliminary experiment (Table 1), it was probably because the atomized water spray lasted only for 5 s in the second experiment compared with 60 s in the first experiment. Conidia growing epiphyllously on the leaf surface of *Poa ampla* were also released by atomized water sprays. In contrast, wind generated by dry compressed air did not dislodge the conidia from the slide cultures nor from the *P. ampla* leaves. Evidence concerning liberation of *Neotyphodium* conidia from the leaves of a host plant by water wash has been reported by Dugan *et al.* (2002). They found the leaf washes from *H. brevisubulatum* subsp. *violaceum* infected by *Neotyphodium* sp. growing and sporulating on the epidermis of the host leaves resulted in colonies of *Neotyphodium* developing on nutrient media. However, the Dugan *et al.* (2002) study did not focus on the method of dissemination of *Neotyphodium* epiphyllic conidia and they did not make comparisons between dry air *versus* atomized water spray in liberation and dispersal of the conidia.

We have SEM documentation of slime coats on some *Neotyphodium* conidia from the *Neotyphodium–Hordeum* and *–Poa ampla* associations. The slime coats are clearly originating from conidia, but are variable in extent (F.D., unpubl.; M.T., unpubl.). Conidial slime coats in fungi may be important in water dispersal (Gregory 1973; Ingold 1953, 1971; Madden 1992; Stepanov 1935). Water currents, rain-splash, drip-splash, and mist pick-up mechanisms may be involved in liberation and dispersal of conidia with slime coats (Lacey 1986; Webster 1980). Western & Cavett (1959) showed that many more conidial spores of *Epichloë typhina* produced on the stromata were released and recovered on trap slides when stromata were subjected to a current of air carrying atomized water than when a similar current of dry air was used. Davies (1959) also showed that the spores of *Verticillium albo-atrum* could be detached from conidiophores only by atomized water droplets. Asexual spores of many other fungal species from the order *Hypocreales* also have been documented to be water disseminated (Bandyopadhyay *et al.* 1991; Chaverri & Samuels 2003; Cross & Jacobs 1969; Mantle 1988; Sutton 1980; Tjamos 1988; Webster 1980). Slime coats analogous to those of the *Neotyphodium–Hordeum* and *–Poa ampla* associations represent a plausible mechanism to explain why water is essential to detachment of conidia.

Little is known of the significance of the epiphyllous stage of *Neotyphodium* endophytes to survival of the fungi. Although it has been hypothesized that epiphyllous conidia might be responsible for the distribution of *N. tembladerae* in multiple grass species in South America, and multistrain infection of single grass plant of *Brachypodium sylvaticum* by *Epichloë sylvatica* (Cabral *et al.* 1999; Meijer & Leuchtmann 1999). In addition, hybridization by somatic fusion of hyphae is a process that is common in fungi (Webster 1980). Some researchers (Chung & Schardl 1997b; Schardl & Craven 2003; Schardl *et al.* 1994; Tsai *et al.* 1994) have indicated that hybridization by somatic fusion of endophyte hyphae apparently could occur between *Epichloë* species. Several asexual grass endophytes are hypothesized to have arisen from such hybridizations (Moon *et al.* 2000, 2002, 2004). Presence of epiphyllous mycelia and conidia of asexual endophytes on the surfaces of some grass species might facilitate this hybridization.

In this study, several other fungal species from the phyllosphere of *P. ampla* were isolated by both compressed air and atomized water spray (Table 4). The results suggest that phyllosphere fungi of *P. ampla* may be dominated by *Cladosporium*

Table 2 – Release of *Neotyphodium* conidia from slide cultures

Treatment	No. of colonies[a]
Air	0
Atomized water spray	61.33 ± 24.01

a Data represent the mean of three individual experiments ± S.D.

Table 3 – Release of *Neotyphodium* conidia from *Poa ampla* leaves

Treatment	No. of colonies[a]	
	Neotyphodium sp.	Other fungi
Air	0	1.83 ± 0.98
Atomized water spray	17.83 ± 27.76	380.83 ± 162.53

a Data represent the mean of three individual experiments ± S.D.

Table 4 – Number of colonies and frequencies of fungi isolated from *Poa ampla* leaves by air and atomized water spray

Fungal isolate	Air		Atomized water spray	
	No. of colonies	Frequency (%)	No. of colonies	Frequency (%)
Acremonium sp.	0	0	30	1.25
Cladosporium sp.	9	90.00	1859	77.72
Neotyphodium sp.	0	0	107	4.47
Penicillium sp.	0	0	121	5.06
Trichoderma sp.	0	0	1	0.05
Yeasts	1	10.00	173	7.23
Mycelia sterilia	0	0	101	4.22

sp. during certain seasons. Moy *et al.* (2000) also found that *Cladosporium* was a significant colonizer of leaves of *P. ampla*. In addition, *Cladosporium* species usually dominate among the filamentous fungi inhabiting the leaf surfaces of other members of *Poaceae* (Di Menna 1971; Smedegaard-Petersen & Tolstrup 1985; Tolstrup & Smedegaard-Petersen 1984) and the aerial surfaces of many flowering plants throughout temperate regions (Dickinson 1971; Lee & Hyde 2002).

In conclusion, the present study demonstrates that conidia of *Neotyphodium* sp. from *P. ampla* may be disseminated by air currents containing water, but are not disseminated solely by air currents. *P. ampla* is native to the Pacific Northwest and northern intermountain area of North America (Duell 1985). The grass is adapted to adverse sites and climates including extreme temperature and humidity conditions (Marble *et al.* 1985). It would seem unlikely that this *Neotyphodium* sp. would allocate scarce resources to produce epiphyllous conidia if they were of no benefit to the fungus. It is possible that epiphyllous conidia may be a means whereby largely asymptomatic *Neotyphodium* endophytes may spread from plant to plant in grass populations. Clearly, more research is required to determine the role of conidia in the life cycle of this and other *Neotyphodium* species. In general, the life cycle of endophytic fungi of grasses is not fully understood.

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